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# Cold-induced changes in the energy coupling and the UCP3 level in rodent skeletal muscles

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**Abstract**

The mechanism of thermoregulatory uncoupling of respiration and phosphorylation in skeletal muscles has been studied. It is found that 24 h cold exposure results in (i) a 3-fold increase in the amount of UCP3 protein in rat skeletal muscle mitochondria, and (ii) pronounced lowering of the membrane potential in isolated rat or mouse skeletal muscle mitochondria. The decrease in membrane potential is reversed by adding bovine serum albumin. Cold exposure is also found to sensitize the membrane potential to the uncoupling action of added fatty acid (laurate). After laurate addition, the recoupling effects of GDP and carboxyatractylate decrease whereas that of albumin increases in mitochondria from cold-treated rats or mice. Changes similar to those induced by cold can be initiated by the in vivo addition of thyroxine. Cold exposure does not affect energy coupling in liver mitochondria. The possible involvement of UCP3 isoforms in nucleotide-sensitive and -insensitive uncoupling is discussed. 

*Keywords:* Muscle mitochondria; Thermoregulatory uncoupling; Thyroxine; Uncoupling protein-3; Fatty acid

## 1. Introduction

Thermoregulatory uncoupling of respiration and phosphorylation has been discovered in the skeletal muscle mitochondria of pigeons exposed to a repeated short-term cold stress [1,2]. This observation was confirmed when the effects of short-term cooling of cold-adapted mice [3] and long-term cold acclimation of fur seals [4] on skeletal muscle mitochondria were studied. Later thermoregulatory uncoupling was demonstrated in intact rat diaphragm muscle [5] and in brown fat, the mammalian tissue specialized in heat production (for reviews, see [6–10]). In brown fat mitochondria, a protein was found called uncoupling protein-1 (UCP1) or thermogenin which is responsible for the fatty acid-mediated increase in the $H^+$ permeability of the inner mitochondrial membrane [11]. UCP1 activity was found to be inhibited by GDP [9,10]. Cold adaptation was shown to stim-

Abbreviations: $\Delta\Psi$, transmembrane electric potential difference; BSA, bovine serum albumin; cAtr, carboxyatractylate; EGTA, ethylene glycol-bis(2-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; SF6847, 3,5-di(*tert*-butyl)-4-hydroxybenzylidenemalononitrile; TMPD, *N*,*N*′-tetramethyl-*p*-phenylenediamine; UCP, uncoupling protein

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ulate transcription of UCP1 mRNA [12] and its translation [13].

UCP1 is absent in tissues other than brown fat (for reviews, see [14,15]). Hence, it cannot be responsible for thermoregulatory uncoupling in skeletal muscles. Nevertheless, in this tissue uncoupling was shown to be also mediated by fatty acids [8,16]. The problem seemed to be solved when it was found that fatty acid-linked uncoupling can be carried out by mitochondrial anion carriers structurally similar to UCP, namely ATP/ADP antiporter [8,17], aspartate/glutamate antiporter [18], dicarboxylate carrier [19] and phosphate carrier [20].

A new possibility to explain the above relationships arose when uncoupling proteins homologous to UCP1 were discovered. It was reported that in a great majority of tissues UCP mRNAs can be revealed. In mammals, five types of such mRNAs were described encoding UCP2 [21], UCP3L, UCP3S [22,23], UCP4 [24] and brain mitochondrial carrier protein 1 (BMCP1) [25]. The UCP2 mRNA is present in all tissues but hepatocytes, whereas the UCP3 mRNA is present in skeletal muscle and brown fat, and the UCP4 and BMCP1 mRNA mainly in brain. UCP2 [23,26] and UCP3 [27] mRNAs were shown to increase when an animal is exposed to cold. A similar cold effect was observed in plants where UCP was also found [28,29].

According to Lin et al. [27], exposure of rats to 5°C for 24 h induced a 3-fold enhancement of the UCP3 mRNA level in skeletal muscles. At longer exposures, this level lowered whereas that of UCP2 mRNA increased 2-fold. In this paper, we used the same scheme of the treatment of rats and mice to study the possible contribution of various mitochondrial systems to thermoregulatory uncoupling in skeletal muscles. In the same rat muscle samples, the level of the UCP3 protein was measured.

## 2. Materials and methods

### 2.1. Treatment of animals

To study the effect of cold, rats or mice previously kept at 23°C were exposed individually to 5°C for 24 h. To produce hyperthyroidism, rats received for 5 days water supplemented with thyroxine (1 mg $ml^{-1}$). The hypothyroid state was obtained by adding mercazolyl (2.5 mg/ml) to water for 4 days.

### 2.2. Isolation of mitochondria

The skeletal muscle or liver mitochondria from the control (23°C) or cold-exposed (5°C) rats or mice were isolated as described elsewhere [30]. When indicated, BSA (2 mg $ml^{-1}$) was added to the medium for homogenization, washing and suspending of mitochondria and to the incubation mixture. The same procedure was used to obtain rat liver mitochondria.

### 2.3. Western blot analysis

30 μg of mitochondria purified from rat muscle as described above were dried under vacuum and resuspended in 10 μl of the loading buffer (50% glycerol, 5% SDS, 0.5 M Tris-HCl pH 6.8, 2.5% bromophenol blue). The samples were subjected to SDS-PAGE on a 12% polyacrylamide gel, and transferred to a nitrocellulose membrane. After blocking the membrane with a PBS buffer containing 0.1% Tween and 2% non-fat dry milk (Bio-Rad No. 170-6404), UCP3 protein was detected using a rabbit polyclonal antibody against 14 amino acids located at the C-terminus of the human UCP3 protein (RDI-UCP3CabrX) at a concentration of 1 μg/ml in PBS, 0.1% Tween, 2% milk. The membrane was washed with PBS, 0.1% Tween, then hybridized with a goat anti-rabbit peroxidase-labelled secondary antibody (Santa Cruz, SC-2004), diluted 1/1000 with PBS, 0.1% Tween, 2% milk. The cytochrome oxidase protein was detected as above using a monoclonal antibody specific for cytochrome oxidase subunit IV (Molecular Probes, A-6432), diluted 1/1000 with PBS, 0.1% Tween, 2% milk and a goat anti-mouse peroxidase-labelled secondary antibody (Santa Cruz, SC-2005), diluted 1/1000 with PBS, 0.1% Tween, 2% milk. Chemiluminescence and quantification of signals were performed as mentioned above. The signals were detected by chemiluminescence using a standard ECL kit (Amersham RPN2106), and developed on a Hyperfilm ECL (Amersham RPN3103H).

Several lines of evidence indicate that the signals obtained are UCP3-specific: interaction with mouse recombinant UCP3, expected size, i.e. 34 kDa, en-

richment in mitochondria vs. homogenate and absence in liver.

### 2.4. Measurement of respiration and membrane potential

Respiration was measured by a Clark oxygen electrode and a Rank Brothers polarograph. Membrane potential was monitored with safranin O [31]. The 555–523 nm light absorption difference was measured by an Aminco DW 2000 spectrophotometer. In some experiments, a fluorimeter was used to the same purpose (495 nm, excitation; 586 nm, emission). For all the measurements, the incubation mixture contained 250 mM sucrose, 10 mM MOPS, pH 7.4, 1 mM EGTA, 2 μM rotenone, 3 μg $ml^{-1}$ oligomycin, 3 mM potassium phosphate, 8 μM safranin O, 0.12 mM TMPD, 5 mM ascorbate, and mitochondria, 1 mg protein $ml^{-1}$.

Mitochondrial proteins were measured by the biuret method.

### 2.5. Chemicals

EGTA, rotenone, GDP, ADP, MOPS, fatty acid-free BSA, laurate and lauryl sulphate were from Sigma, and glutamate from Serva.

## 3. Results

Measurement of UCP3 in rat skeletal muscles by Western blot revealed a 3-fold increase in the level of this protein by the cold treatment. In the cold-exposed rats, the UCP3 amount proved to be equal to 301% (S.E.M., 79%; $P < 0.01$). As a standard we employed cytochrome oxidase, since its level was found to be constant during the 24 h cold exposure (Fig. 1).

In Fig. 2 one can see typical ΔΨ responses of skeletal muscle mitochondria from control (A), cold-exposed (B) and thyroxine-treated (C) rats to various uncouplers and recouplers (medium for isolation and incubation of mitochondria contained no BSA). One can see that addition of BSA increased the ΔΨ level. Subsequent addition of laurate decreased ΔΨ, the effect being reversed by recouplers (GDP, cAtr, and glutamate) and by the binding of laurate with BSA.

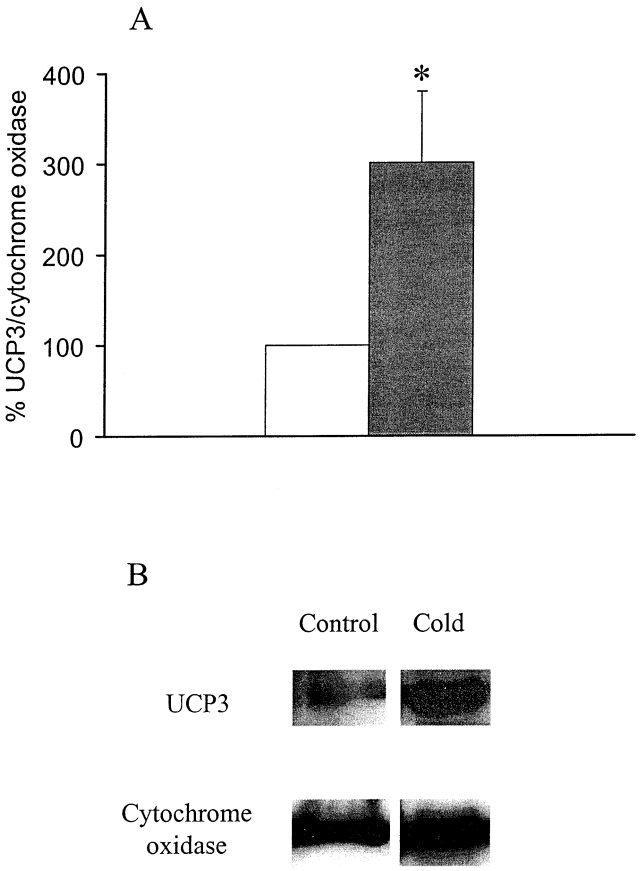


Fig. 1. Cold exposure increases the UCP3 protein level in rat muscle. (A) Level of UCP3 in hind limb mitochondria of control (empty column) and 24 h cold-exposed (shaded column) rats. Photodensitometric comparison of 34 kDa signals on Western blots was performed as described in Section 2. The results are expressed as percentage ± S.E of the mean control value taken as 100%. $n = 6$. $^*P < 0.01$. (B) Typical UCP3 and cytochrome oxidase signals.

Uncoupler SF6847 added after the second BSA addition completely dissipated ΔΨ. 5 μM ADP was found to effectively substitute for 0.2 mM GDP as a recoupler (not shown, see also [30]).

Comparison of Fig. 2A and B shows that in mitochondria from control and cold-exposed rats cold exposure resulted in (i) a strong decrease in the initial ΔΨ level observed before the first BSA addition; (ii) a strong rise in recoupling by the first portion of BSA; (iii) sensitization of ΔΨ to laurate; (iv) a decrease in recoupling by GDP and cAtr; (v) elevation of recoupling by the second portion of BSA added after laurate, GDP, cAtr and glutamate.

Experiments also showed that BSA added before laurate at a concentration of 1 mg $ml^{-1}$ was as ef-

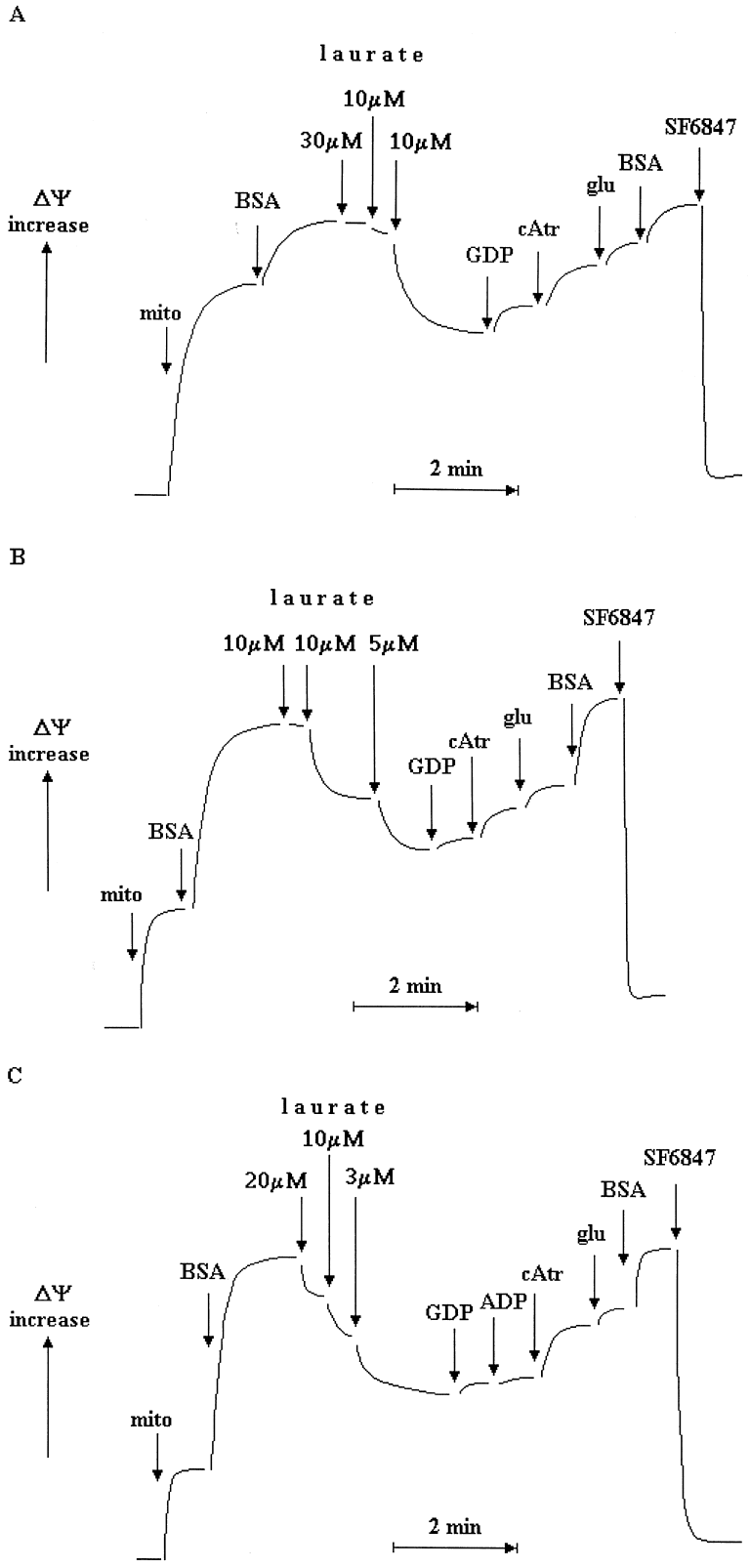


Fig. 2. Effect of various uncouplers and recouplers on the membrane potential of skeletal muscle mitochondria from control (A), cold-exposed (B) and hyperthyroid (C) rats. Mitochondria were isolated without BSA. Additions: mitochondria (mito) (1 mg $ml^{-1}$), BSA (2 mg $ml^{-1}$), 0.2 mM GDP, 2 μM cAtr, 7 mM glutamate (glu), 60 nm SF6847.

Table 1
Cold exposure of animals increases the recoupling effect of BSA addition to rat and mice skeletal muscle mitochondria isolated without BSA

| Animal | BSA-induced ΔΨ increase (%) | |
|---|---|---|
| | Control | Cold |
| Rat | 41 ± 4.6 | 66 ± 2.2 |
| Mice | 47 ± 1.4 | 74 ± 1.4 |

Mitochondria were added to a medium containing ascorbate and TMPD (see Section 2.4). When a steady state level of safranin O response was achieved, BSA (2 mg $ml^{-1}$) was added. BSA addition resulted in an increase in the safranin O response to a new steady state level which can be taken as maximal since further BSA additions failed to elevate the response. The effect of the first BSA addition was expressed as percentage of the maximal safranin O response.
In each series, seven experiments were done. In both cases, $P < 0.01$ (cold against control).

Table 3
Effect of cold exposure of rats on the action of laurate and various recouplers on the ΔΨ level in skeletal muscle mitochondria

| Subsequent additions | Control ($n = 19$) | Cold ($n = 15$) |
|---|---|---|
| *Uncoupler* | | |
| Laurate ($C_{1/2}$, μM) | 50 ± 1.4 | 25 ± 1.1* |
| *Recouplers (recoupling effect, %)* | | |
| GDP | 18 ± 1.8 | 8 ± 0.7* |
| cAtr | 41 ± 1.3 | 28 ± 2.5* |
| Glutamate | 18 ± 1.0 | 19 ± 0.9 |
| BSA | 23 ± 1.3 | 45 ± 2.6* |

*$P < 0.01$.
Mitochondria were isolated with BSA. For other conditions, see Fig. 2.
100% recoupling means complete reversal of uncoupling caused by 50 or 25 μM laurate added to mitochondria from control or cold-exposed rats, respectively.

fective as the 5-fold lower BSA amount used in Fig. 2. A higher laurate sensitivity of mitochondria from the cold-treated animals could still be revealed in the presence of a high concentration of BSA (not shown in the figure).

Statistical analysis of the ΔΨ of skeletal muscle mitochondria from control, cold-exposed and thyroxine-treated rats, as well as comparisons between rat and mice are shown in Table 1–4. Mice skeletal muscle mitochondria isolated without BSA have, like rat mitochondria, a much lower ΔΨ after cold exposure (not shown). Addition of BSA caused a higher recoupling in mitochondria of cold-treated animals (Table 1). Effects similar to that of cooling were shown to be caused by hyperthyroidism (Fig. 2C and Table 2). On the other hand, the hypothyroid state shifted the measured parameters in the opposite direction (Table 2).

Tables 3 and 4 show the influence of cold exposure on laurate uncoupling in skeletal muscle mitochondria from rats and mice, respectively. In both cases, mitochondria were isolated and incubated with BSA to avoid interference of cold-induced changes in endogenous fatty acids [16]. Three effects of cold proved to be common for both species, namely (i) sensitization of mitochondrial ΔΨ to laurate uncoupling, (ii) a decrease in the recoupling action of GDP and (iii) an increase in that portion of laurate uncoupling which cannot be recoupled by GDP, cAtr and glutamate but is abolished by BSA. However, the influence of cold proved to be smaller in mice than in rats, possibly due to the fact that 24 h cold exposure, which was optimal for UCP3 expression in rats [27], proved not to be optimal in mice.

Table 2
Recoupling effect of BSA on skeletal muscle mitochondria from hyper- and hypothyroid rats

| Animal | BSA-induced ΔΨ increase (%) | $P$ |
|---|---|---|
| Control | 46 ± 2.1 | |
| Hyperthyroid | 61 ± 3.7 | < 0.02 |
| Hypothyroid | 38 ± 1.7 | < 0.05 |

For conditions, see Table 1.

Table 4
Effect of cold exposure of mice on the action of laurate and various recouplers on the ΔΨ level in skeletal muscle mitochondria

| Subsequent additions | Control ($n = 10$) | Cold ($n = 8$) |
|---|---|---|
| *Uncoupler* | | |
| Laurate ($C_{1/2}$, μM) | 54 ± 1.5 | 32 ± 1.4* |
| *Recouplers (recoupling effect, %)* | | |
| GDP | 17 ± 1.2 | 11 ± 0.6* |
| cAtr | 31 ± 0.8 | 31 ± 0.8 |
| Glutamate | 17 ± 1.1 | 18 ± 0.8 |
| BSA | 34 ± 1.0 | 39 ± 1.5** |

*$P < 0.01$.
**$P < 0.02$.
Mitochondria were isolated with BSA. For other conditions, see Fig. 2 and Table 3.

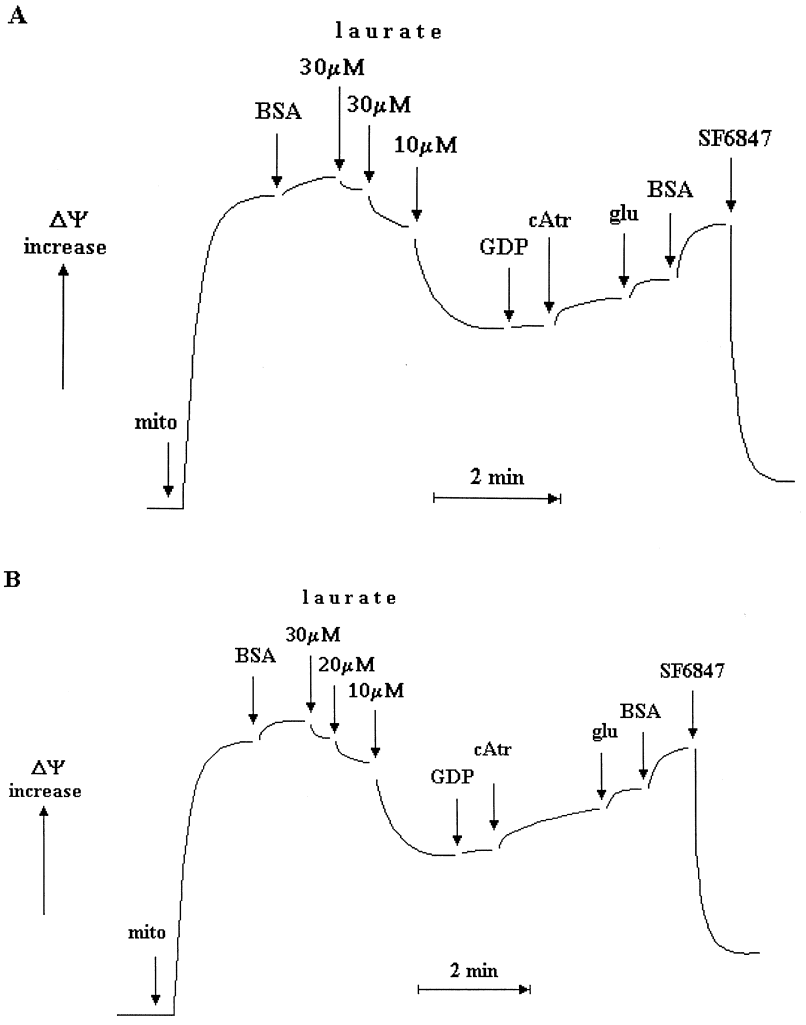


Fig. 3. Effect of various uncouplers and recouplers on the membrane potential of liver mitochondria from control (A) and cold-exposed (B) rats. Mitochondria were isolated without BSA. For other conditions, see Fig. 2.

GDP, cAtr and glutamate added before the first portion of BSA induced recoupling in a fashion similar to that revealed in the presence of laurate, i.e. the relative contribution of GDP in cooled animals was lowered (not shown).

In rat liver mitochondria, subsequent additions of cAtr, glutamate and BSA were shown to effectively reverse laurate uncoupling whereas 0.2 mM GDP was ineffective. None of the above-listed effects of cold exposure in skeletal muscles was observed in liver (Fig. 3).

In line with our preceding paper [30], lauryl sulphate caused an uncoupling which was resistant to GDP but sensitive to cAtr, glutamate and BSA. This was shown in skeletal muscle mitochondria from both control and cold-exposed rats. Cold exposure increased the sensitivity of mitochondria to lauryl sulphate and enlarged BSA recoupling, the latter effect being smaller than with laurate (not shown).

In all the above experiments, we used ascorbate and TMPD as the respiratory substrate. This allowed us to avoid any complications related to regulation at the level of dehydrogenases. On the other hand, the respiratory control ratio is known to be much lower in such a system compared with a NAD-linked substrate or succinate. Nevertheless, some stimulation of respiration by laurate was observed, which was absent when recouplers were added (not shown).

## 4. Discussion

The results described in this paper are in line with previous observations [1–5,16] indicating that cold exposure increases fatty acid-mediated thermoregulatory uncoupling in skeletal muscle mitochondria. In fact, the amount of UCP3 (uncoupling protein specific for skeletal muscle and brown fat, i.e. for tissues involved in regulatory heat production) strongly increases by cold treatment. Such an increase is accompanied by a strong $\Delta\Psi$ decrease in muscle mitochondria that become more sensitive to fatty acid-induced uncoupling.

The cold exposure-induced increase in UCP3 observed in this study is in good agreement with data of Lin et al. [27] who showed a cold-dependent 3-fold rise in the UCP3 mRNA level in rat skeletal muscles. Elevation of the UCP3 content may, in principle, be responsible for some of the $\Delta\Psi$ decrease and for the higher effect of BSA observed in the skeletal muscle mitochondria from cold-exposed animals.

As was previously shown by our group [33], a 48 h exposure of rats to 6°C (which is known to result in a 4.3-fold increase in UCP2 mRNA in the heart muscle [23]) results in (i) some uncoupling in heart muscle mitochondria and (ii) the appearance of a recoupling effect of 0.5 mM GDP, the same amount of CDP being ineffective in the control animals. GDP had no influence on the membrane potential level and cAtr failed to substitute for GDP.

In contrast to the latter observation, GDP added to skeletal muscle mitochondria before cAtr showed recoupling, whereas after cAtr it was ineffective [30]. Thus, one might assume that the GDP-sensitive uncoupling mechanism in skeletal muscle mitochondria is not related to UCP3. One may suggest that in these mitochondria GDP interacts, in fact, with the ATP/ADP antiporter. This explains why (i) much smaller concentrations of ADP substitute for GDP as recoupler and (ii) cAtr is much more efficient than GDP and prevents the GDP effect [30]. The inefficacy of GDP in liver may be explained if we assume that the liver ATP/ADP antiporter has a higher specificity in binding of purine nucleotides than its muscle isoform. On the other hand, in skeletal muscle mitochondria the decrease in the GDP- and cAtr-induced recoupling under cooling might be accounted for by an increase in the amount of a form of UCP3 which is GDP-resistant. It is remarkable that hyperthyroidism, which like cold treatment strongly increases the UCP3 mRNA level [34], influences mitochondria in a way similar to that of cooling (cf. Fig. 1A–C).

The above assumption that UCP3 in cold-exposed rats is GDP-resistant requires some discussion. According to Jaburek and coworkers [32], human UCP3L is competent in fatty acid-mediated $H^+$ conductance when it is inserted into proteoliposomal membranes. This conductance is partially inhibited by GDP ($C_{1/2} = 0.5$ mM). We have found a recoupling effect of GDP on skeletal muscle mitochondria at concentrations 10-fold lower than that used by Jaburek and coworkers [32]. An increase in the GDP concentration failed to induce any additional recoupling in mitochondria from cold-exposed rats (not shown). It might be suggested that in cold-exposed animals the purine nucleotide inhibition is prevented by some modification of the UCP3 nucleotide binding site which strongly lowers or even abolishes its nucleotide affinity.

It is also noteworthy that in man [22] and mouse [35] there are two UCP3 mRNAs, i.e. long (L) and short (S). In man, the UCP3 mRNA isoforms correspond to proteins of 312 (L) and 275 (S) amino acids, the sixth transmembrane domain being absent in the S isoform. In mouse these values are 308 and 214 amino acids (the fifth and sixth transmembrane domains are absent in the S isoform). The sixth domain is known to be required for purine nucleotide binding. This means that UCP3S, in contrast to UCP3L, cannot be inhibited by GDP. As was shown by Hinz et al. [36], UCP3S expressed in yeast has a stronger uncoupling activity than UCP3L (see, however, [37]).

One may speculate that an increased thermogenesis upon cold exposure in skeletal muscles is mediated by both an increase in the UCP3 level and a decrease in the nucleotide inhibition of UCP3 activity. It is a decrease in the nucleotide inhibition of UCPs that is one of the possible explanations for a quite recent and very interesting finding of Kozak and coworkers [38]. They reported on cold resistance of mice obtained by hybridization of two cold-sensitive strains of UCP1 knockout animals. Neither UCP2 nor UCP3 mRNA levels were elevated in skeletal muscles and brown fat of the hybrid mice com-

pared to the parental strains. Perhaps the activity, rather than the amount, of the muscle UCPs changes in response to the disappearance of UCP1 which is the major uncoupling mechanism in brown fat. Adaptive activation of systems alternative to the knockout one is not surprising if we take into account the complexity of the organization of higher organisms. For example, the absence of obvious defects in oxygen supply in tissues of myoglobin knockout mice was initially erroneously interpreted as evidence against the $O_2$ transporting function of this protein [39]. However, more careful investigation revealed that in these mice small but predictable changes occurred in some parameters, apparently compensating for such defects [40] (for discussion, see [41]).

The absence of a strong influence of the UCP3 knockout on thermoregulation in mice [42,43] might be accounted for in a similar way. In these studies, it was revealed that a proton leak and respiration in state 4 were decreased in the skeletal muscle mitochondria isolated from the UCP3-deficient animals, which is in good agreement with our data on cold exposure and with the data of Clapham et al. [44] that overexpression of the UCP3 gene causes a decrease in energy coupling. However, the body temperature at cold exposure was only insignificantly lower in the UCP3 knockout mice in comparison with the control [42,43]. Further studies are required to identify alternative mechanisms of thermoregulation.

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